FINAL REPORT

Study Title

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY

Test Substances

O
K
M
C
D
S

Reference Substance

H

Authors

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Study Completion Date

January 31, 2006

Performing Laboratory

Institute for In Vitro Sciences, Inc.
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Study Number

05AG40-AG45, 05AE40.350064

Laboratory Project Number

4228

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STATEMENT OF COMPLIANCE

The Bovine Corneal Opacity And Permeability Assay With Two Time Exposures and Optional Histology of the test substances, O, K, M, C, D, S, and H, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

> The identity, strength, purity and composition or other characteristics to define the test substances have not been determined by the testing facility.
>
> The stability of the test substances under the test conditions has not been determined by the testing facility and is not included in the final report.
>
> Analyses to determine the uniformity, concentration, or stability of the test substance mixtures, if applicable, were not performed by the testing facility.

_________________________________ ___________________________

John W. Harbell, Ph.D. Date
Study Director

QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Two Time Exposures and Optional Histology

Study Number: 05AG40-AG45, AE40.350064

Study Director: John Harbell, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLP Standards (40 CFR 792 and 40 CFR 160) and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director	Reported to Management
Protocol and Initial Paperwork	17-Oct-05	17-Oct-05	31-Oct-05
Isolation of the Corneas	02-Nov-05 08-Nov-05	07-Nov-05 09-Nov-05	12-Nov-05 12-Nov-05
Histology Evaluation – Positive Control and 05AG43, 10 Minute Exposure Time	06-Jan-06	11-Jan-06	11-Jan-06
Final Report and Data	16-Jan-06	16-Jan-06	31-Jan-06

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Amanda K. Ulrey, RQAP-GLP
Quality Assurance

Date

SIGNATURE PAGE

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY

Initiation Date:	October 14, 2005
Completion Date:	January 31, 2006
Sponsor:	
Sponsor's Representative:	
Testing Facility:	Institute for In Vitro Sciences, Inc. 21 Firstfield Road, Suite 220 Gaithersburg, MD 20878
Archive Location:	Institute for In Vitro Sciences, Inc. Gaithersburg, MD 20878
Study Director:	____________________ John W. Harbell, Ph.D. Date
Laboratory Management:	Greg Mun, B.A.
Principle Investigator (slide preparation): Pathology Associates, A Charles River Company	David A. Hodge HT (ASCP)
Histological Evaluation performed by:	John W. Harbell, Ph.D.

TEST SUBSTANCE RECEIPT

IIVS Test Substance Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
05AG40	O	clear colorless non-viscous liquid	9/30/05	room temperature
05AG41	K	fine white powder	9/30/05	room temperature
05AG42	M	clear light yellow non-viscous liquid	9/30/05	room temperature
05AG43	C	clear yellow non-viscous liquid	9/30/05	room temperature
05AG44	D	cloudy white semi-viscous suspension	9/30/05	room temperature
05AG45	S	clear dark blue non-viscous liquid	9/30/05	room temperature
05AE40	H	clear colorless non-viscous liquid	8/1/05	room temperature

* - Protected from exposure to light

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY

INTRODUCTION

The Bovine Corneal Opacity and Permeability Assay (BCOP) was used to assess the potential ocular irritancy of the test substances to isolated bovine corneas. Bovine corneas, obtained as a by-product from freshly slaughtered animals, were mounted in special holders and exposed to the test substances. An *in vitro* score was determined for each of two exposure times tested for each test substance based on the induction of opacity and permeability (to fluorescein) in the isolated bovine corneas.

The purpose of this study was to evaluate the potential ocular irritancy of the test substances as measured by changes in opacity and permeability (to fluorescein) in isolated bovine corneas. The laboratory phase of this study was conducted from November 2, 2005 to January 8, 2006 at the Institute for In Vitro Sciences, Inc. Three corneas were treated with each test substance at two exposure times of 3 and 10 minutes. Based on changes in corneal opacity and permeability (relative to the control corneas), an *in vitro* score was determined at each exposure time.

MATERIALS AND METHODS

Bovine Eyes

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals (J.W. TREUTH & SONS, Inc., Baltimore, MD). The eyes were excised and then placed in Hanks' Balanced Salt Solution, containing Penicillin/Streptomycin (HBSS), and transported to the laboratory on ice packs. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas was initiated.

Preparation of Corneas

The eyes were grossly examined for damage and those exhibiting defects were discarded. The tissue surrounding the eyeball was carefully pulled away and the cornea was excised such that a 2 to 3 mm rim of sclera was present around the cornea. The isolated corneas were then stored in a petri dish containing HBSS until they were mounted in a corneal holder. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber was then positioned on top of the cornea and the screws were tightened. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium (EMEM) without phenol red, containing 1% fetal bovine serum and 2 mM L-glutamine (Complete MEM). Each corneal holder was uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The corneal holders were incubated at 32 ± 1ºC for a minimum of 1 hour.

Assay Controls

The positive assay control used in this study was neat ethanol (Pharmco). The negative assay control used in this study was sterile, deionized water (Quality Biological).

Test Substance Preparation

As instructed by the Sponsor, one entire packet of the test substance, K, was diluted in 1.25 gallons of deionized water. All of the other test substances were administered to the test system without dilution.

Test Substance pH Determination

The pH of each test substance was determined using pH paper (EMD Chemicals Inc./ EM Science). Initially, each test substance was added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, each test substance was added to 0-6 pH paper with 0.5 pH unit increments or 7.5-14 pH paper with 0.5 pH unit increments, to obtain a more accurate pH value. The pH values obtained from the narrower range pH paper are presented in Table 1.

Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the corneas were removed from the incubator. The medium was removed from both chambers and replaced with fresh Complete MEM. The initial opacity was determined for each cornea using a Spectro Designs OP-KIT opacitometer.

Three corneas, whose initial opacity readings were close to the median opacity for all the corneas, were selected as the negative control corneas. The treatment of each cornea was identified with the test substance number written in permanent marker on colored tape, affixed to each holder. The medium was then removed from the anterior chamber and replaced with the test substance, positive control, or negative control.

<u>Method for Testing Liquid or Surfactant Materials</u>

The liquid test substances, O, M, C, D, S, and H, were tested neat. The test substance, K, was diluted in 1.25 gallons of deionized water and applied to the test system. This dilution was performed by someone not involved in the assay so that the produce name was not revealed to the assay technicians or Study Director. An aliquot of 750 µL of the test substance, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. Due to its viscous nature, the test substance, D, was administered directly onto the exposed cornea using a positive displacement pipette. A group of three corneas was incubated in the presence of each test substance at 32 ± 1ºC for 3 minutes. A second set of three corneas was incubated in the presence of each test substance at 32 ± 1ºC for 10 minutes. A set of three corneas was incubated in the presence of the positive control at 32 ± 1ºC for 10 minutes. A set of three corneas was incubated in the presence of the negative control at 32 ± 1ºC for 10 minutes. After the 3 and 10-minute exposure times, the control or test substance treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control or test substances. The corneas were then given a final rinse with Complete MEM (without phenol red). For the corneas directly exposed to the test substance (without anterior chamber window), the test substance was removed from the treated corneas by rinsing the exposed epithelium of the corneas (special care was taken not to spray the corneas directly) with Complete MEM (with phenol red). The chamber window was returned to the chamber when most or all of the test substance had been removed. The rinsing process continued in the same manner as the positive and negative control corneas. The anterior chamber was refilled with fresh Complete MEM (without phenol red) and an opacity measurement was performed. The corneas were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was filled with fresh Complete MEM and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at 32 ± 1ºC. At the end of the 90-minute incubation period, the medium was removed from the posterior chamber and placed into tubes numbered corresponding to chamber number. Aliquots of 360 µL from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm (OD_{490}) was determined using a Molecular Devices *V*max kinetic microplate reader. If the OD_{490} value of a control or test substance sample was 1.500 or above, a 1:5 dilution of the sample was prepared in Complete MEM (to bring the OD_{490} value within the linear range of the platereader). A 360 µL sample of each 1:5 dilution was transferred to its specified well on the 96-well plate. The plate was read again and the final reading was saved to a designated print file.

Fixation of Corneas

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual prelabeled tissue cassette containing a biopsy sponge. The endothelial surface of each cornea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the corneal tissue for at least 24 hours. The fixed corneas will be stored up to one year.

Histological Evaluation

As instructed by the Sponsor, a histological evaluation was not performed for the test substances, O, K, M, D, and S.

The fixed corneas treated with test substances, C and H on 11/02/05, were transferred to Pathology Associates, A Charles River Company (Frederick, MD) for embedding, sectioning, and staining. Each cornea was paraffin-embedded, bisected, and the two halves mounted in the paraffin block so that a section of each half could be cut and placed on a single slide. Each slide was then stained with hematoxylin and eosin. Slides were returned to IIVS for evaluation.

Presentation of Data

Opacity Measurement: The change in opacity for each cornea (including the negative control corneas) was calculated by subtracting the initial opacity reading from the final opacity reading. These values were then corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of each cornea for that treatment condition.

Permeability Measurement: The mean OD_{490} for the blank wells was calculated. The mean blank OD_{490} was then subtracted from the raw OD_{490} of each well (corrected OD_{490}). Any dilutions that were made to bring the OD_{490} readings into the linear range of the platereader (OD_{490} should be less than 1.500), had each diluted OD_{490} reading multiplied by the dilution factor. The final corrected OD_{490} of the test substances and the positive control was then calculated by subtracting the average corrected OD_{490} of the negative control corneas from the corrected OD_{490} value of each treated cornea:

$$\text{Final Corrected } OD_{490} = (\text{raw } OD_{490} - \text{mean blank } OD_{490}) - \text{average corrected negative control } OD_{490}$$

The mean OD_{490} value of each treatment group was calculated by averaging the final corrected OD_{490} values of the treated corneas for that treatment condition.

The following formula was used to determine the *in vitro* score:

In Vitro Score = Mean Opacity Value + (15 x Mean OD_{490} Value)

Criteria for Determination of a Valid Test

The BCOP assay was accepted when the positive control (ethanol) caused an *in vitro* score that fell within two standard deviations of the historical mean.

RESULTS AND DISCUSSION

Bovine Corneal Opacity and Permeability Assay

Table 1 summarizes the opacity, permeability, and *in vitro* score for each test substance. Table 2 summarizes the opacity, permeability, and *in vitro* score for the positive control. Since the results of the positive control fell within two standard deviations of the historical mean (within a range of 39.9 to 64.5), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

The following classification system was established by Sina et al.[1] based on studies with a wide range of test materials tested under standard conditions (e.g., 10-minute exposure for liquids). This scoring scale was initially developed for application to industrial hygiene labeling of pharmaceutical intermediates. While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of materials or alternate exposure times.

In Vitro Score:

from 0 to 25	= mild irritant
from 25.1 to 55	= moderate irritant
from 55.1 and above	= severe irritant

[1]Sina, J.F., Galer, D.M., Sussman, R.G., Gautheron, P.D., Sargent, E.V., Leong, B., Shah, P.V., Curren, R.D., and Miller, K. (1995) A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates. **Fundamental and Applied Toxicology** 26:20-31.

Table 1
BCOP Results of the Test Substances

Assay Date	IIVS Test Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD_{490} Value	*In Vitro* Score	pH
11/2/05	05AG40	O	Neat	3 minutes	2.3	0.016	2.6	9.5
				10 minutes	6.7	0.037	7.2	
	05AG41	K	*	3 minutes	0.0	-0.002	0.0	4.0
				10 minutes	0.3	0.000	0.3	
	05AG42	M	Neat	3 minutes	7.7	0.509	15.3	12.5
				10 minutes	32.0	1.582	55.7	
	05AG43	C	Neat	3 minutes	6.0	0.308	10.6	3.0
				10 minutes	18.7	0.736	29.7	
	05AE40	H	Neat	3 minutes	1.0	0.140	3.1	12.0
				10 minutes	2.7	0.745	13.9	
11/8/05	05AG44	D	Neat	3 minutes	67.3	0.008	67.5	0.0
				10 minutes	187.7	0.003	187.7	
	05AG45	S	Neat	3 minutes	2.3	0.460	9.2	1.0
				10 minutes	13.0	0.385	18.8	
	05AE40	H	Neat	3 minutes	0.7	0.279	4.9	12.0
				10 minutes	5.3	1.064	21.3	

*- As instructed by the sponsor, 1 packet of the test substance was diluted in 1.25 gallons of deionized water.

Table 2
BCOP Results of the Positive Assay Control

Assay Date	Positive Control	Conc.	Exposure Time	Mean Opacity Value	Mean OD_{490} Value	*In Vitro* Score
11/2/05	Ethanol	Neat	10 minutes	26.3	1.336	46.4
11/8/05	Ethanol	Neat	10 minutes	34.0	0.985	48.8

Histological Evaluation

Basis for Histological Evaluation

Damage to the epithelial layer is generally the first change seen in the cornea. This is not surprising as the test materials are applied topically to this "unprotected" epithelium. Each "layer" of the epithelium is scored for cell loss or damage. Figure 1 shows the structure of the epithelium from a control cornea (not from this study). Changes to the surface epithelium (squamous and upper wing cell epithelium) in the BCOP assay are usually not predictive of lasting corneal changes *in vivo*. The loss of the squamous and upper wing cell layers in the excised bovine cornea by surfactant-based test substances appears to coincide with mild to moderate damage to the conjunctiva of the rabbit *in vivo*. This type of damage is typically reversible in the rabbit. Many types of surfactants (e.g., sodium lauryl sulfate) are expected to lyse these cells so that they are progressively lost from the epithelial surface. The positive control, ethanol, provides an example of a different type of damage. In this case, the surface squamous epithelial cells are coagulated and fixed in place. The coagulated tissue stains heavily with eosin (see Figure 5). In addition, the wing and basal cells are vacuolated and the adhesion between the basal cells and the basal lamina is disrupted. Such changes can lead to sloughing of the epithelium. Lesions in the deep wing cell and basal cell layers, either by cell lysis or coagulation, are associated with more damage *in vivo*. Much of the available data come from studies on surfactants and show that loss of the bovine corneal epithelium is predictive of some corneal opacity in the rabbit[2].

Special effort has been made to detect changes in the stromal elements of the corneas. Jester[3], Maurer[4,5] and others have shown for a range of chemical classes that depth of injury in the early hours after exposure can be predictive of the eventual degree and duration of the ocular

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[2] Gettings, SD, Lordo, RA, Hintze, KL, Bagley, DM, Casterton, PL, Chudkowski, M, Curren, RD, Demetrulias, JL, DiPasquale, LC, Earl, LK, Feder, PI, Galli, CL, Glaza, SM, Gordon, VC, Janus, J, Kuntz, PJ, Marenus, KD, Moral, J, Pape, WJW, Renskers, KJ, Rheins, LA, Roddy, MT, Rozen, MG, Tedeschi, JP, and Zyracki, J. (1996) The CTFA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test. (Phase III) Surfactant-based formulations. **Food Chemical Toxicology** 34:79-117.

[3] Jester, J.V., Li, H.F., Petroll, W.M., Parker, R.D., Cavanaugh, H.D., Carr, G.J., Smith, B., and Maurer, J.K. (1998) Area and depth of surfactant-induced corneal injury correlates with cell death. **Invest Ophthalmol Vis Sci** 39:922-936.

[4] Maurer, J.K. and Parker, R.D. (1996) Light microscopic comparison of surfactant-induced eye irritation in rabbits and rats at three hours and recovery/day 35. **Toxicologic Pathology** 24:403-411.

[5] Maurer, J.K., Parker, R.D., and Jester, J.V. (2002) Extent of initial corneal injury as the mechanistic basis for ocular irritation: key findings and recommendations for the development of alternative assays. **Regulatory Toxicology and Pharmacology** 36:106-117.

lesions. Epithelial damage alone, in the rabbit cornea, is associated with expected recovery provided the basal lamina is intact. Deep injury to the stroma has more serious consequences. Stromal damage may be manifest by several types of changes within the tissue. These may include swelling within the stromal collagen matrix and loss/damage to the keratocytes. These changes do not necessarily occur together. Stromal swelling may be detected by the presence of vacuole-like "holes" in the organized collagen matrix. Their appearance suggests that liquid has entered the matrix expanding space between the fibers. Examples of these vacuoles may be seen in Figure 8 where the positive control exposure has induced some stromal swelling. The depth and degree of vacuolization can be indicative of the degree of injury to the cornea and/or penetration of the test substance into the tissue. Loss of the effective epithelial or endothelial barrier will allow water (medium) to enter the stroma and produce the collagen matrix vacuolization (swelling). Viable epithelium or endothelium will not only retard entry of the water but actively transport it out of the stroma. As the viability of epithelium declines, both the barrier and transport capacities are reduced. Thus, the amount of water accumulating in the upper stroma reflects the damage to the epithelium. When the full epithelial layer is lost over a section of stroma, stromal swelling can increase the stromal thickness by 50% or more over the control corneal stroma. Such changes are often observed when corneas treated with severe irritants are incubated for extended periods after exposure. Loss of the endothelium can also lead to appreciable deep stromal swelling. The loss may result from test substance penetration or mechanical damage. Mechanical damage tends to be more focal (patchy) in nature while the true toxic response to the test substance exposure tends to extend across much of the cornea. When focal (mechanical) damage is present, the majority of the collagen matrix vacuolization will be located in the deep stroma (just above Descemet's Membrane). In contrast, test substance-induced toxicity to the endothelium should include both epithelial as well as endothelial damage so that collagen matrix vacuolization will be observed throughout the stroma. Such extreme damage may increase the stromal thickness two fold over the control corneal stroma thickness.

In vivo, the inflammatory response that follows deep initial injury can, itself, lead to permanent lesions through "scar" collagen deposition or neovascularization in the corneal stroma. The authors cited above have suggested that the damaged keratocytes are involved in initiating this inflammatory process. Classic inflammatory changes (e.g., inflammatory cell infiltration) are possible only in the presence of active circulation through the limbus. Since the isolated cornea has no source of inflammatory cells, the potential for test substance-induced inflammation is judged by the changes in the extracellular matrix and particularly the keratocytes. Some forms of damage are more easily recognized than are others. Necrotic cell death, as might follow exposure to a strong alkaline, would be quite apparent since the cellular components rapidly break down. More subtle damage could also lead to a delayed cell death and release of inflammatory mediators. Nuclear changes (vacuolization [swelling], punctate chromatin condensation, pyknosis or karyorrhexis) are signs of this process. Cytoplasmic changes can also be informative. Vacuole formation and/or loss of basic elements (mRNA for example) are also indicative of the beginning of the degenerative process. The cell cytoplasm normally stains with both basophilic (hematoxylin) and acidophilic (eosin) stains. When the basic elements are lost, eosinophilic staining predominates. This type of change is reported as cytoplasmic eosinophilia. Harbell and Curren[6] have reported that mechanical removal of the corneal epithelium leads to marked stromal swelling (marked collagen matrix vacuolization). The keratocytes in the zone of marked swelling undergo changes as well. The nuclei become enlarged and the cytoplasmic staining is very eosinophilic. Thus, when test substance-treated corneas show only this

[6] Harbell, J.W. and Curren, R.D. (2005) Using histological evaluation to enhance the Bovine Corneal Opacity and Permeability (BCOP) assay. **ALTEX** 42(Special Issue):236.

type of keratocyte change (with stromal swelling), the change may be the result of the stromal swelling rather than direct action of the test substance on the cells.

Since depth of injury (both extracellular and cellular) to the stroma is important in the analysis, a means to describe the depth of injury is necessary as part of this analysis. Determining this depth is not always a straightforward process. Because of the topical application of the test substance to the epithelium, one would expect that exposure to the stroma would progress from the area just under Bowman's Layer down through the stroma to Descemet's Membrane. There is no external inflammatory process *in vitro*, so one might also expect the progression of damage to follow the progression of exposure. That means that damage to the stroma should first appear close to Bowman's Layer. As the damage to the stroma increases, deeper layers might be involved. This implies that one would want to express damage as progressing from the anterior (Bowman's Layer) to the posterior (Descemet's Membrane) and express it as a fraction of the total stromal depth involved. However, collagen matrix vacuolization can (and often does) increase total stromal thickness. Thus, measurements of the depth of a stromal lesion can be complicated by the change in overall stromal thickness. To account for stromal swelling, this depth is actually estimated from the percentage of the stromal cross section that remained undamaged (starting at the posterior border). For example, a cornea reported to show collagen matrix vacuolization to 30% depth would mean that 70% of the cross section of that cornea (starting at Descemet's Membrane) did not show vacuolization. For this report, depth of stromal damage is reported as the <u>percentage of the normal corneal depth (cross-section) involved</u>, starting from the anterior border (Bowman's Layer). It should be clearly understood that the percentage of the stromal depth is only an estimate developed by evaluating several fields in each cornea (where possible). The values are, by necessity, approximations of an average depth to which the lesion extended (e.g., collagen matrix vacuolization). It would be unwise to try to compare small differences in the reported depth. Rather, one should focus on broader bands of depth (e.g., upper, middle and deep stroma).

Photomicrographs of the epithelium and stroma are intended to illustrate the degree of damage at the indicated depth. Where little or no damage to the stroma was detected, the area directly under Bowman's Layer was photographed since that is where one would expect to see damage if it had occurred. Images were prepared using a Spot Insight Digital Camera and Spot 4.0.8 software (Diagnostic Instruments, Inc., Sterling Heights, MI). The color balance of the images was corrected to better represent the colors that would be seen through the microscope. Photomicrographs taken of the epithelium often overexposed the stroma. This leads to the impression that the stroma is more damaged (e.g., vacuolated) than it really is. Stromal changes are better represented in micrographs where the stroma is the central feature of the image.

With the digital camera and associated software, it is possible to measure distances within the captured image. This feature can be used to measure corneal thickness directly. Such measurements require a true cross-section of the cornea (i.e., a perpendicular section relative to the corneal surface plane). In some cases, a true cross-section is not available. The Descemet's Membrane thickness is used to estimate how close the section is to a true cross section. The appearance of an unusually thick membrane suggests that the section was cut at a tangent to the true cross section (or a very old animal). If the measured corneal section did not appear to be a true cross section, the observation was noted. Even in a true cross section, the isolated cornea is not of uniform thickness but rather shows peaks and valleys. Therefore, an effort has been made to select "representative" cross-sections that are neither extreme "peaks" nor "valleys" for measurement of corneal thickness. An example of such a measurement is seen in Figure 4. The values obtained should be considered "representative" of the treatment group rather than strict quantitative measures. Many more measurements would be required to provide a quantitative comparison. In some cases, the treated corneas will have lost most of their epithelium or it has been compromised (i.e., ethanol treatment). In those cases, the stromal thickness of the treated corneas should be compared to the stromal thickness of the negative control-treated corneas.

Histological Evaluation

The negative control corneas were treated for 10 minutes with sterile, deionized water (slides B8916-B8918). The negative control-treated epithelium was composed of three layers. The basal cell layer was a well-formed, columnar-cell region directly attached to the Bowman's Layer. The basal cells were always tightly attached to each other. Several layers of wing cells covered the columnar basal layer. In both of these layers, the cell nuclei showed diffuse chromatin without clear nucleoli. Rare mitotic figures were seen in the basal layer. The squamous layer was flattened with limited cytoplasm and highly condensed nuclei (Figure 2).

The stromal elements showed well-organized collagen matrix fibers with dispersed keratocytes. Keratocyte nuclei showed a range of morphologies from moderate sized (smaller than the epithelial nuclei) with diffuse basophilic staining to narrow, elongated and condensed with dark basophilic staining. Cytoplasmic staining, when it was visible, was moderately basophilic. Rare cells, with eosinophilic cytoplasmic staining, were observed. Collagen bundles were generally parallel and well ordered (Stroma just under Bowman's Layer, Figure 3).

The Descemet's Membrane was prominent. The endothelial layer could be seen in most sections and was reasonably well maintained.

A cross section of the negative control showing the general thickness of the whole cornea and stroma is provided in Figure 4.

The positive control corneas (slides B8919-B8921), treated for 10 minutes with 100% ethanol, showed the classic pattern of squamous layer coagulation (darkening) and marked vacuolization in the wing and basal cell nuclei (Figure 5). The loss of adhesion between the basal cells (cell to cell) and the basal lamina was marked. The epithelium was probably not viable at the time of fixation. Overall, the positive control-treated corneas were thicker than the negative control-treated corneas (Figure 6). In the stroma directly below Bowman's Layer, the collagen matrix showed slight hypereosinophilic staining suggestive of some coagulation. Below this zone, moderate collagen matrix vacuolization extended past 50% depth. In the upper stroma, there was a decrease in the density of viable keratocytes (Figure 7) as reflected by a marked increase in the frequency of keratocytes showing nuclear vacuolization or other forms of abnormal chromatin condensation (Figure 8). In the stroma below mid depth, the keratocytes showed a moderate increase in the frequency of cells with enlarged nuclei (Figure 9). The endothelial cells were generally intact (similar to the negative control-treated corneas).

Table 3
Histological Observations on the Test Article -Treated Corneas

IIVS Number	Sponsor's Designation	Observations	Figure #
05AG43 Slides B8940-B8942	C, neat, 3-minute exposure, 120-minute post-exposure, 11/02/05	Epithelium: The surface squamous epithelium was coagulated. The cells just below the coagulated layer were disrupted so that the coagulated layer had separated from the remaining epithelium in most fields. The deep squamous, wing and basal cells were largely intact but showed an increase in cytoplasmic vacuolization. Nuclear halos (a clear zone around the nucleus) were present in all three test article-treated corneas (and two of the negative control-treated corneas). These are an artifact of processing where the nucleus becomes displaced within the section (Figure 10). Stroma: The test article-treated corneas were thicker than the negative control-treated corneas (Figure 11). Moderate collagen matrix vacuolization extended through the upper half of the stroma and slight vacuolization extended well past mid depth. Keratocyte changes were rather limited. There was a slight to moderate increase in cells with larger nuclei (than in the negative control-treated corneas) in the upper half of the stroma (Figure 12). Endothelium: There were some fields that showed damaged endothelium but there was no appreciable increase in deep collagen matrix vacuolization and so this damage may have occurred either very late in the incubation or after fixation.	10 - 12
05AG43 Slides B8943-B8945	C, neat, 10-minute exposure, 120-minute post-exposure, 11/02/05	Epithelium: All layers of the epithelium showed coagulation and some blanching of the nuclei. The basal cells had separated from the basal lamina and there was precipitate between the basal cells and the Bowman's Layer in many fields (Figures 13 and 14). Bowman's Layer was intact. Stroma: The test article-treated corneas were appreciably thicker than the negative control-treated corneas (Figure 15). Moderate to marked collagen matrix vacuolization extended through the full stromal depth. There was a marked increase in the frequency of keratocytes with	13 - 17

IIVS Number	Sponsor's Designation	Observations	Figure #
		hyper-condensed (and potentially pyknotic) nuclei (Figures 16 and 17). Cytoplasmic eosinophilia was not observed. Endothelium: The endothelium was lost in most fields and there was moderate to marked collagen matrix vacuolization directly above Descemet's Membrane suggesting a loss of endothelial cell function.	
05AE40 Slides B8946-B8948	H, neat, 3-minute exposure, 120-minute post-exposure, 11/02/05	Epithelium: The full squamous layer was lost in most fields. The wing and basal cells were intact (Figure 18). Stroma: The test article-treated corneas were thicker than the negative control-treated corneas (Figure 19). Moderate collagen matrix vacuolization extended through the upper third of the stroma and slight vacuolization extended past mid depth. In the upper half of the stroma, there was a moderate increase in the frequency of keratocytes with abnormally condensed nuclei (Figure 20). The stroma directly above Descemet's Membrane did not show increased vacuolization. Endothelium: The endothelium was generally intact.	18 - 20
05AE40 Slides B8949-B8951	H, neat, 10-minute exposure, 120-minute post-exposure, 11/02/05	Epithelium: The squamous cell layer was lost in all sections. In the many of fields, the wing and basal cells were present but showed abnormal chromatin condensation (punctuate condensation). In many other fields, the basal cells showed marked abnormal nuclear condensation and a breakdown of the cells between the wing and basal layers (Figures 21 and 22). In some cases, the basal cells were completely lost (Figure 23). Bowman's Layer was intact. Stroma: The test article-treated corneas were thicker than the negative control-treated corneas (Figure 24). Moderate collagen matrix vacuolization extended through most of the stromal depth. In most of the fields, there was not an increase in vacuolization directly above Descemet's Membrane. The keratocytes changes were striking. Through most of the stromal depth, the keratocytes showed a marked increase in the frequency of cells with hyper-condensation of	21 - 26

IIVS Number	Sponsor's Designation	Observations	Figure #
		their nuclei (but without appreciable cytoplasmic eosinophilia) (Figures 25 and 26). This pattern of keratocytes changes is consistent with exposure to a reactive chemical. Endothelium: The endothelium was intact and functional in the majority of fields.	

The figures displayed on the subsequent pages of this report are representative hematoxylin and eosin-stained cross-sections presented at the indicated magnification. The black bar, on each micrograph, represents 100 µm. Arrows from the text to the figures are intended to show examples of the lesions mentioned. Not all lesions are marked. The vertical double-headed arrows mark the limits of the epithelium in the appropriate figures.

Figure 1. An example of a control cornea showing the three layers of the epithelium, Bowman's Layer and the upper stroma (magnification 290x)

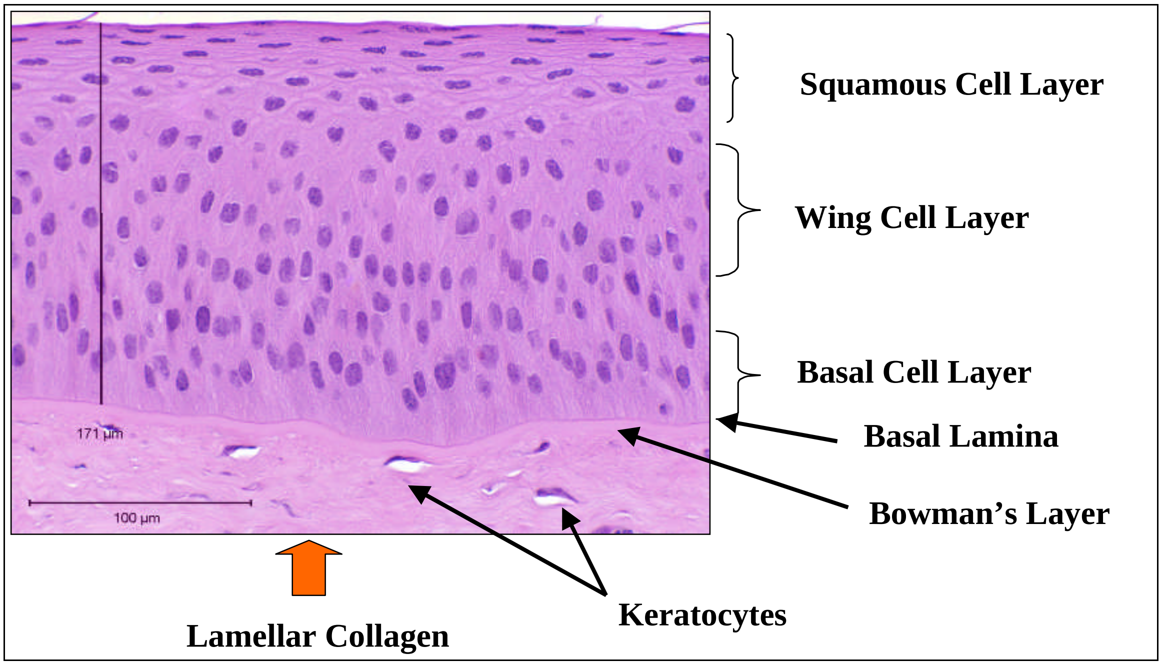


Figure 2. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium (magnification 237x)

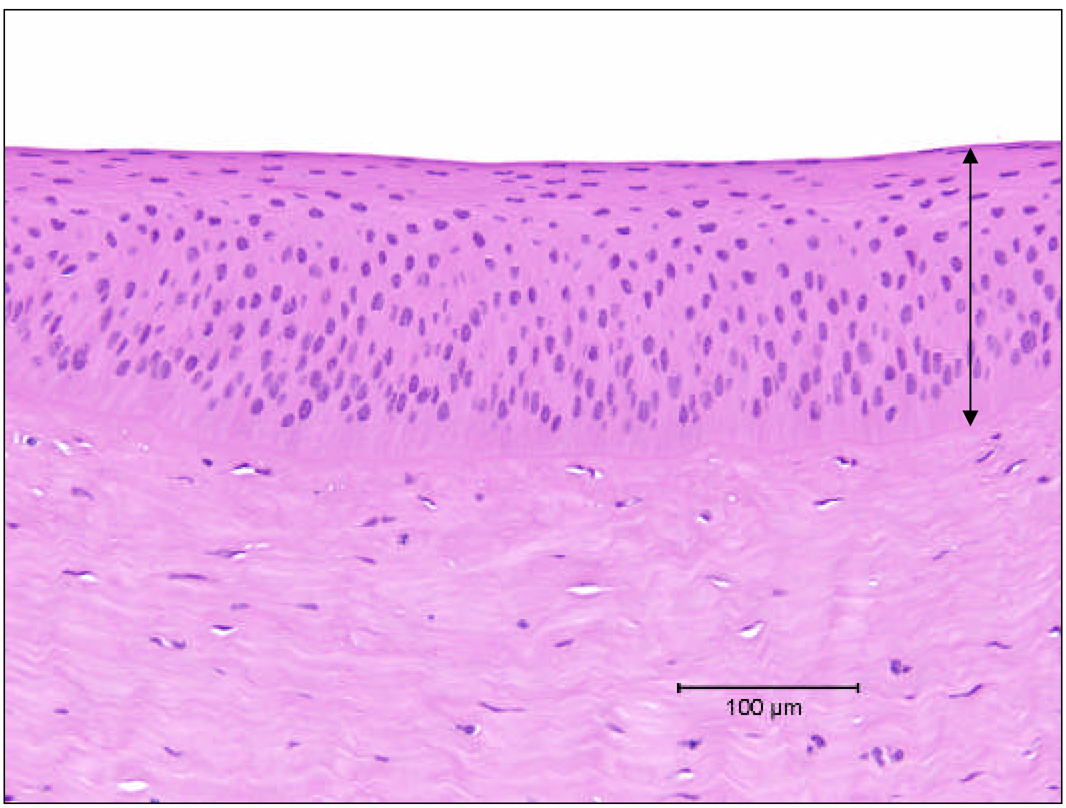


Figure 3. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma directly below Bowman's Layer (magnification 475x)

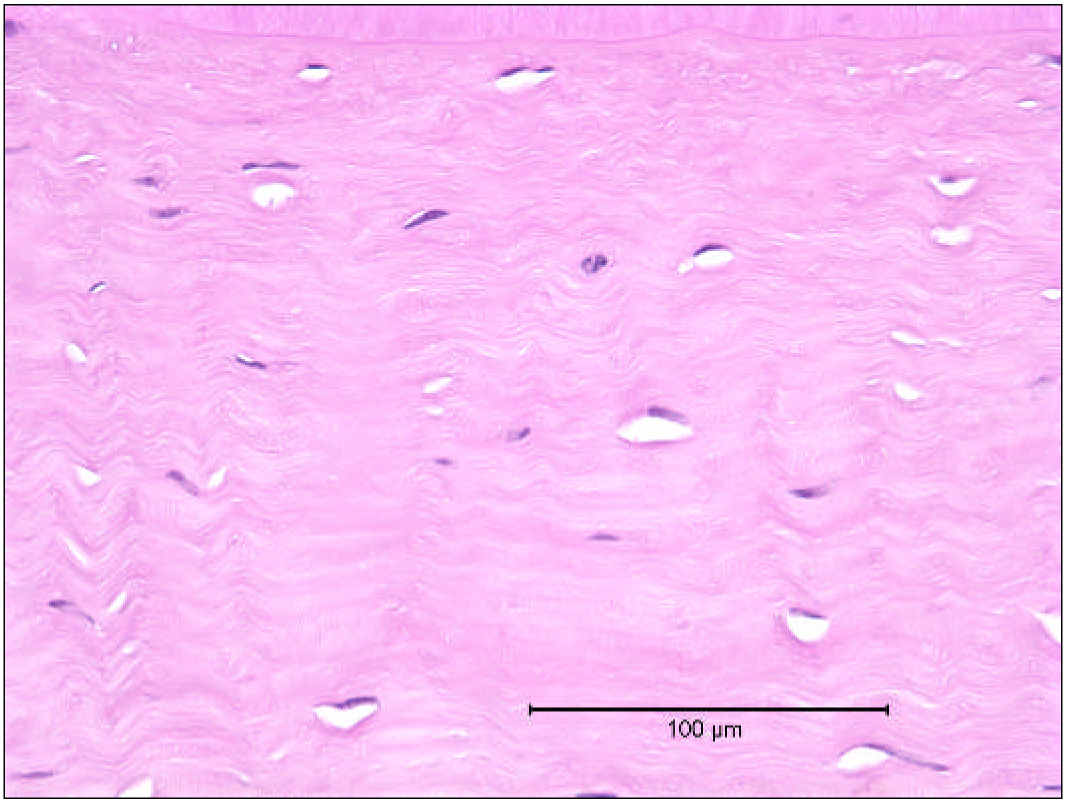


Figure 4. Negative Control (sterile, deionized water), 10-minute exposure, 120-minute post-exposure (11/02/05) - Full thickness (magnification 48x)

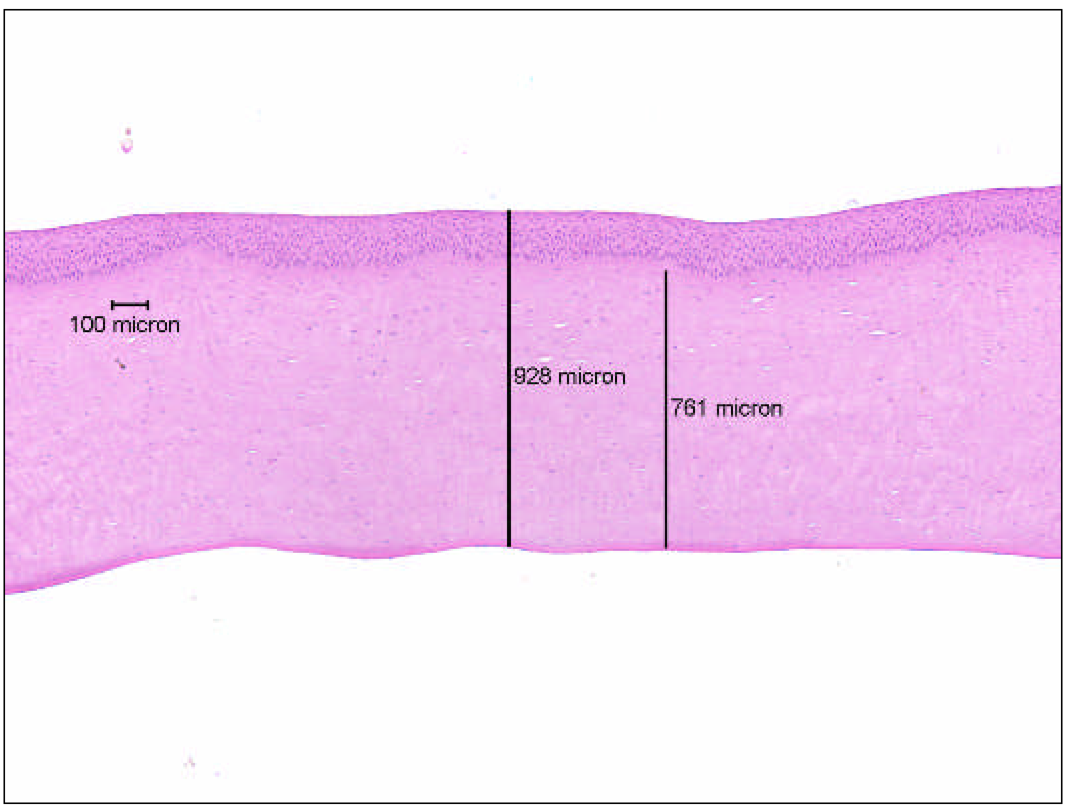


Figure 5. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium (probably not viable at the time of fixation) (magnification 292x)

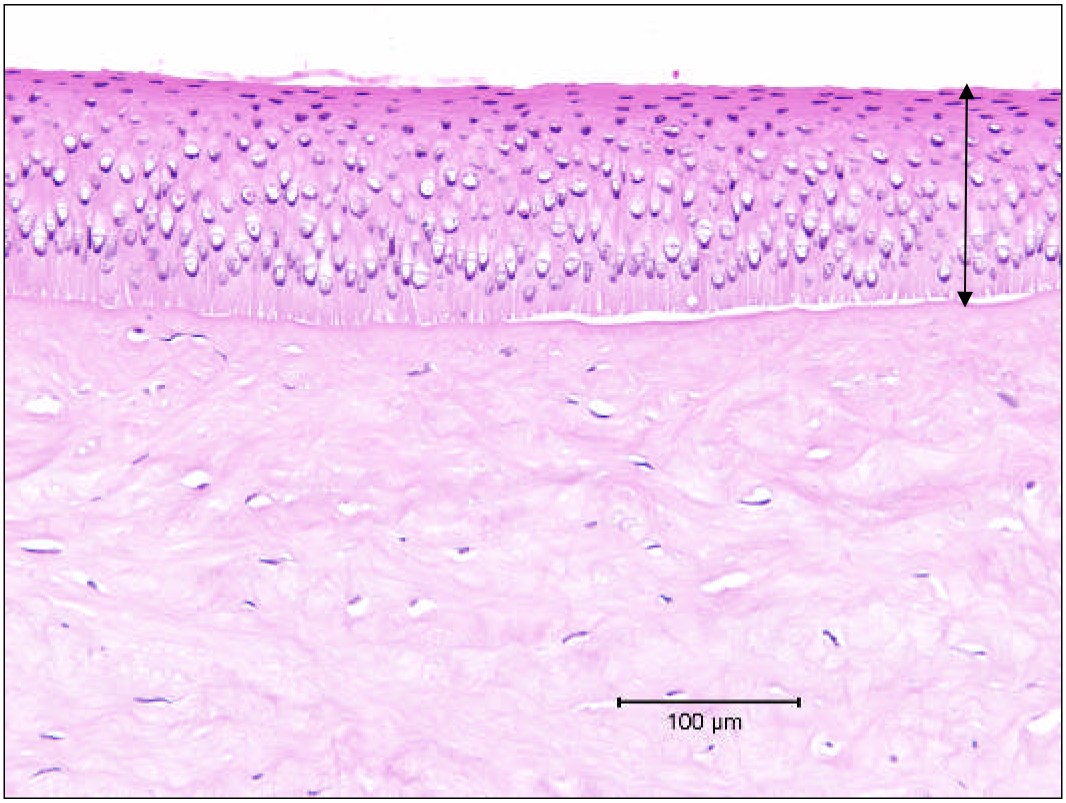


Figure 6. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Full thickness (magnification 48x)

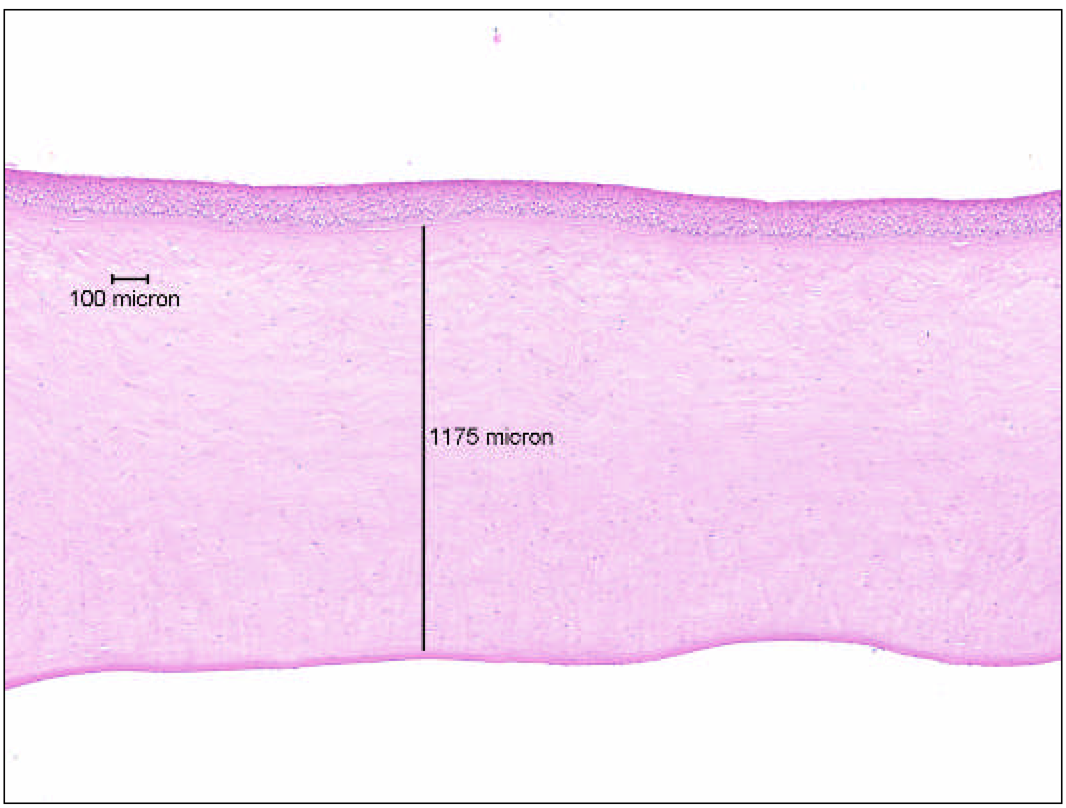


Figure 7. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Upper stroma showing hyperchromic staining in the zone directly below Bowman's Layer and the decrease in the density of viable keratocytes (magnification 237x)

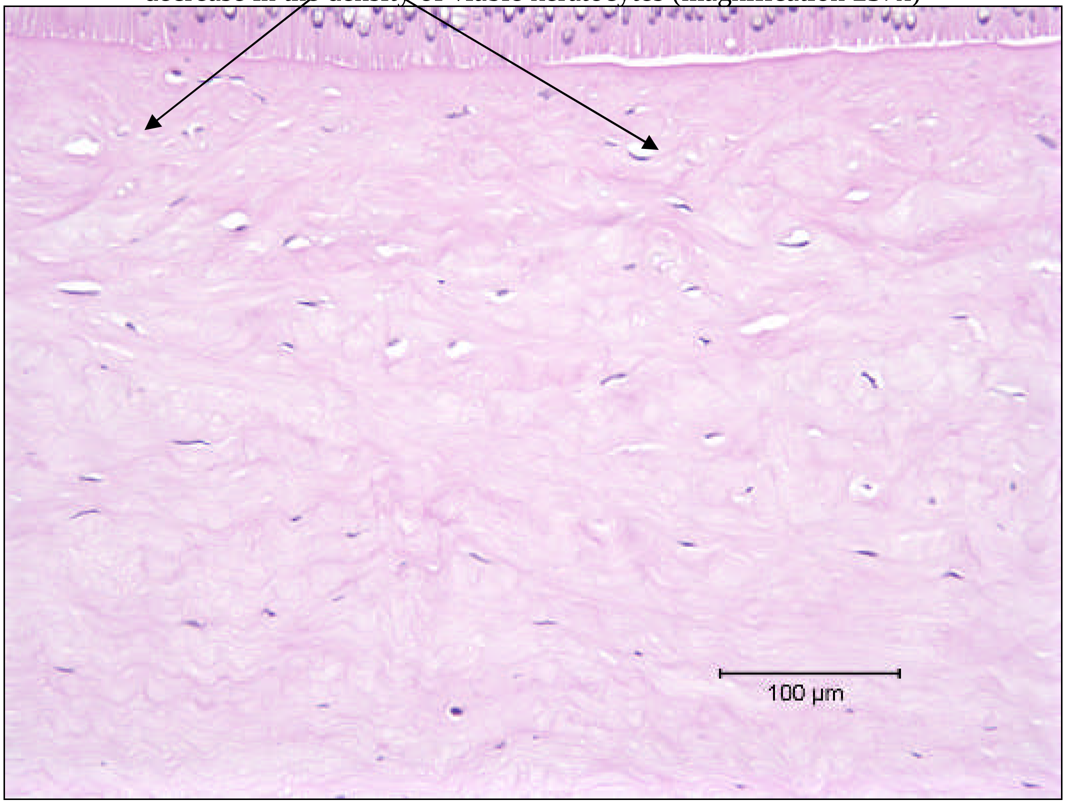


Figure 8. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and an increased frequency of keratocytes with abnormal chromatin condensation (magnification 237x)

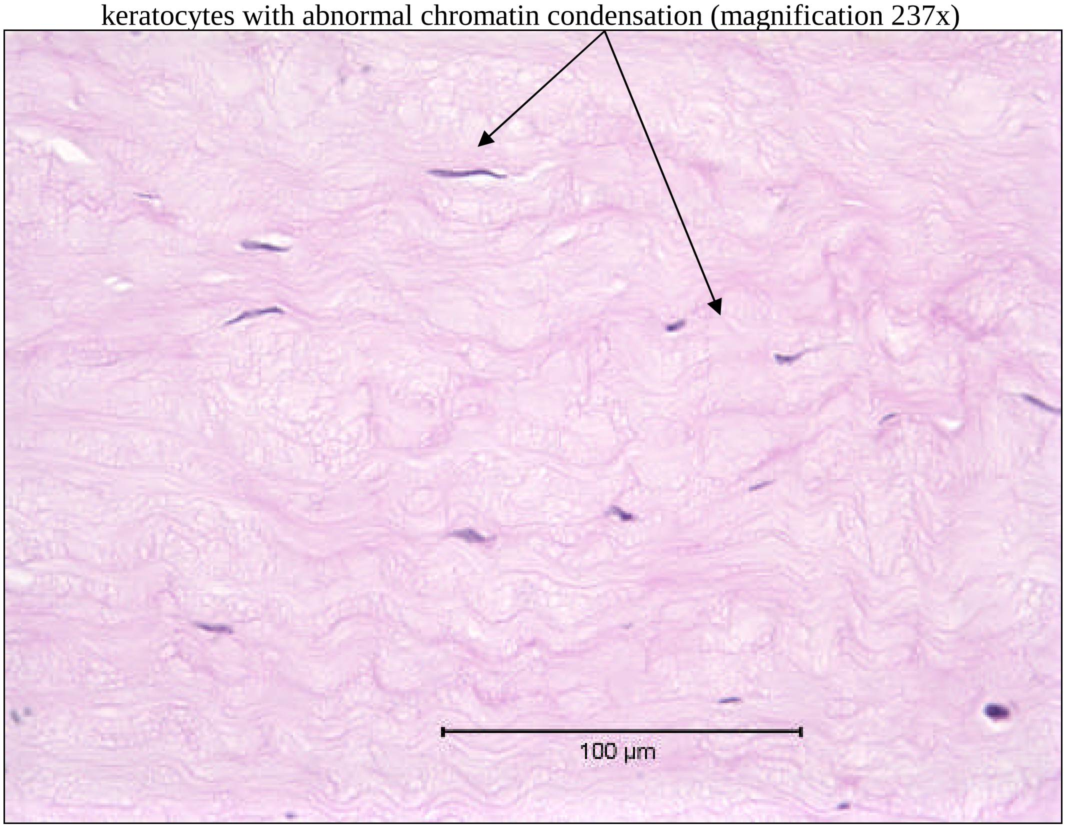


Figure 9. Positive Control (100% ethanol), 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma below mid depth showing keratocyte with nuclear enlargement (magnification 475x)

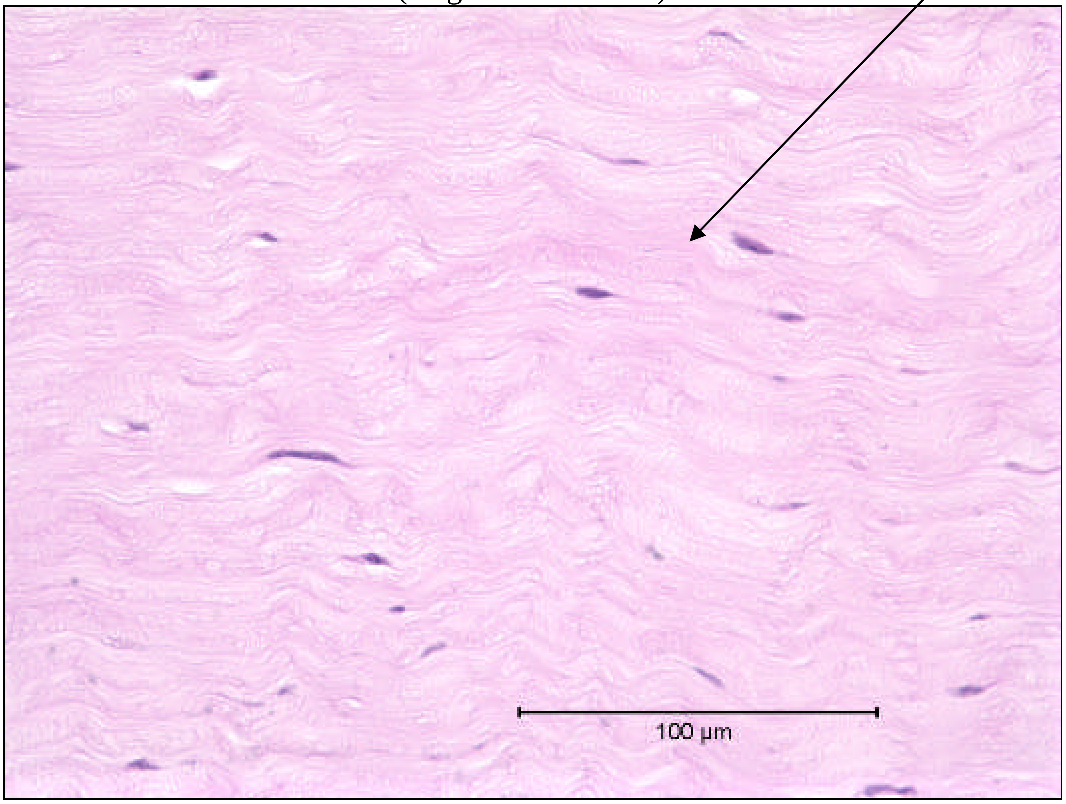


Figure 10. C, neat, 3-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium (note the presence of nuclear halos) (magnification 237x)

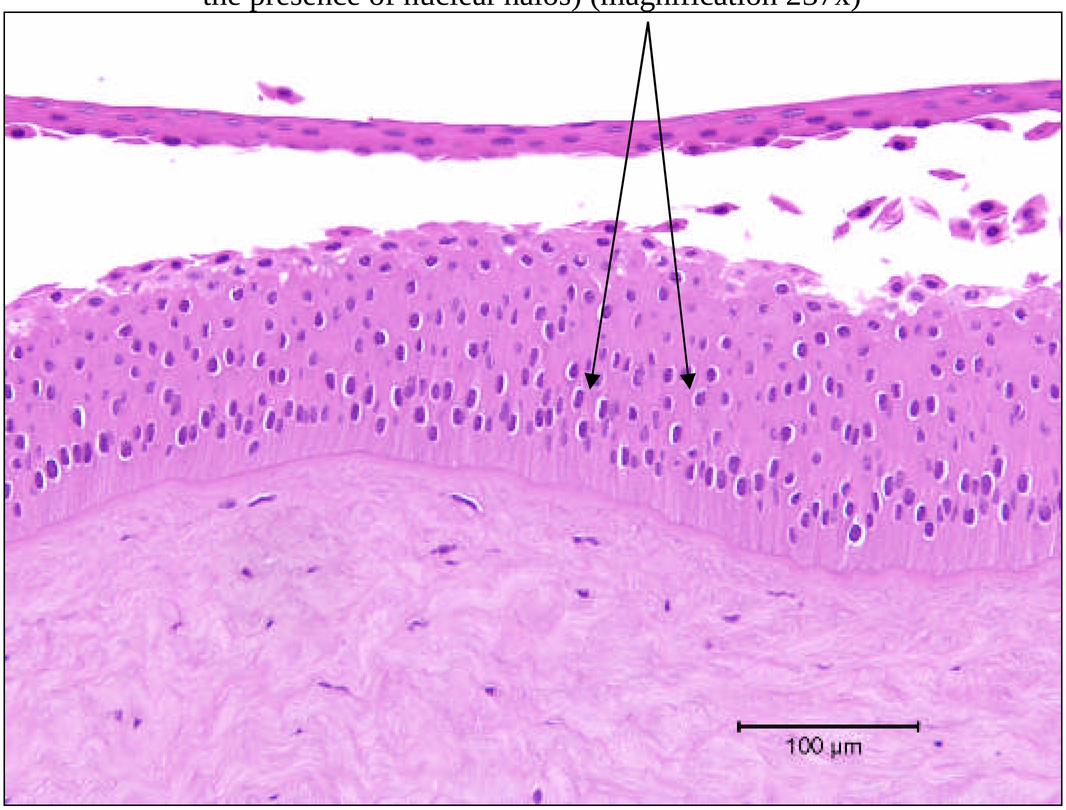


Figure 11. C, neat, 3-minute exposure, 120-minute post-exposure (11/02/05) - Full thickness (magnification 48x)

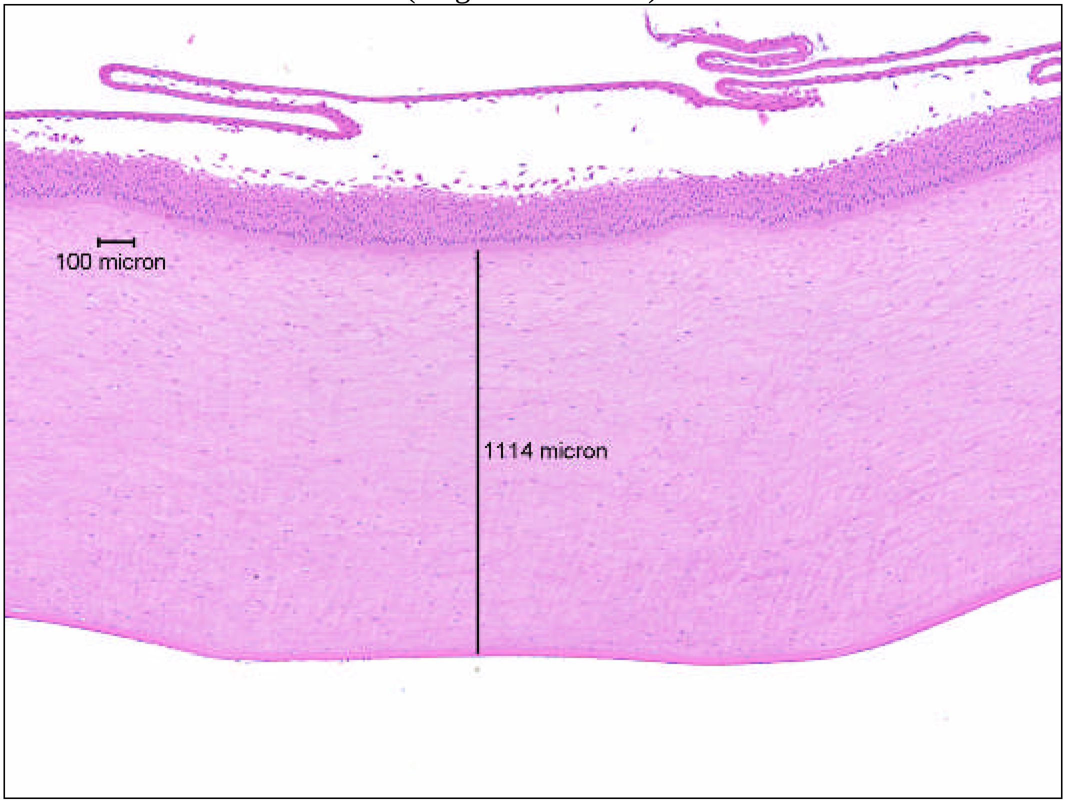


Figure 12. C, neat, 3-minute exposure, 120-minute post-exposure (11/02/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and a slight increase in keratocytes with larger nuclei (magnification 475x)

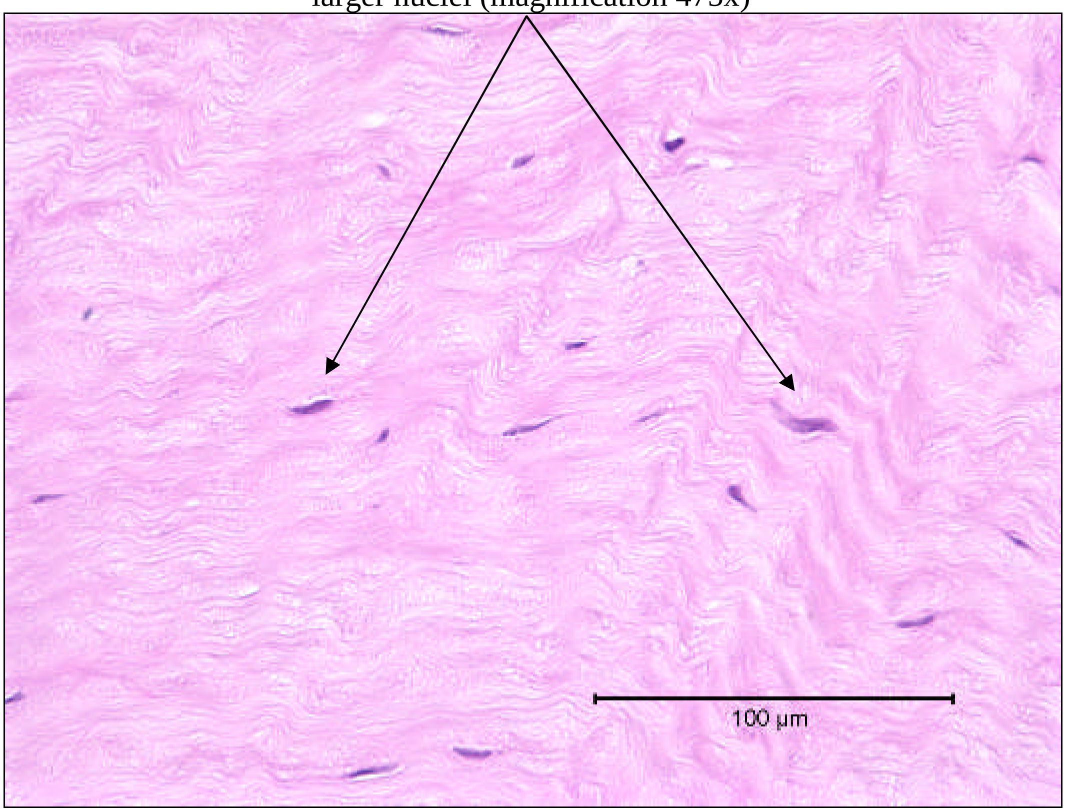


Figure 13. C, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium (probably not viable at the time of fixation)(overview) (magnification 237x)

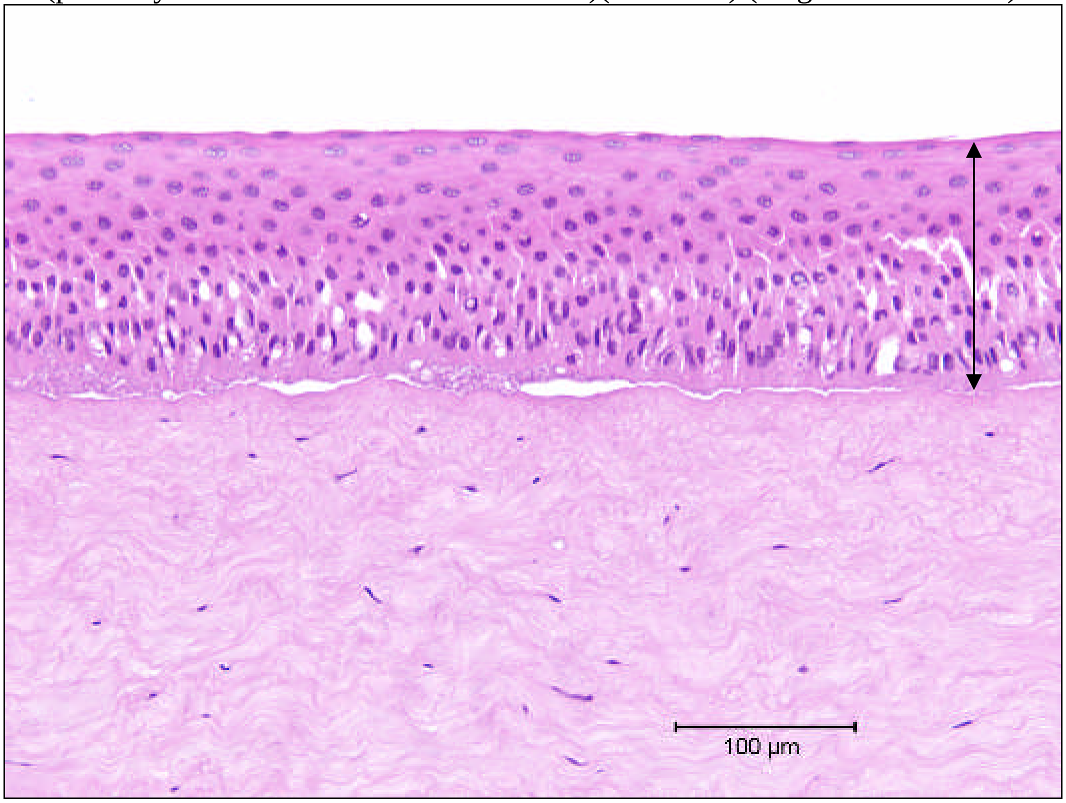


Figure 14. C, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium showing coagulation of all three cell layers and precipitate between the basal cells and Bowman's Layer (magnification 475x)

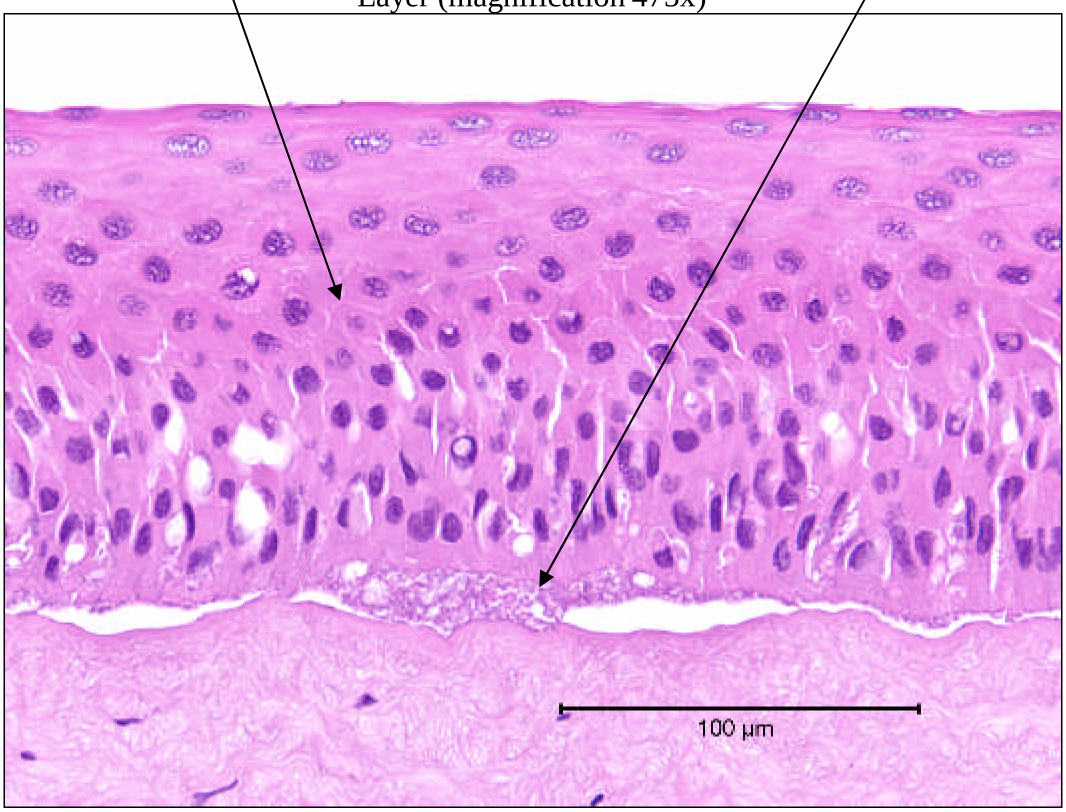


Figure 15. C, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Full thickness (magnification 48x)

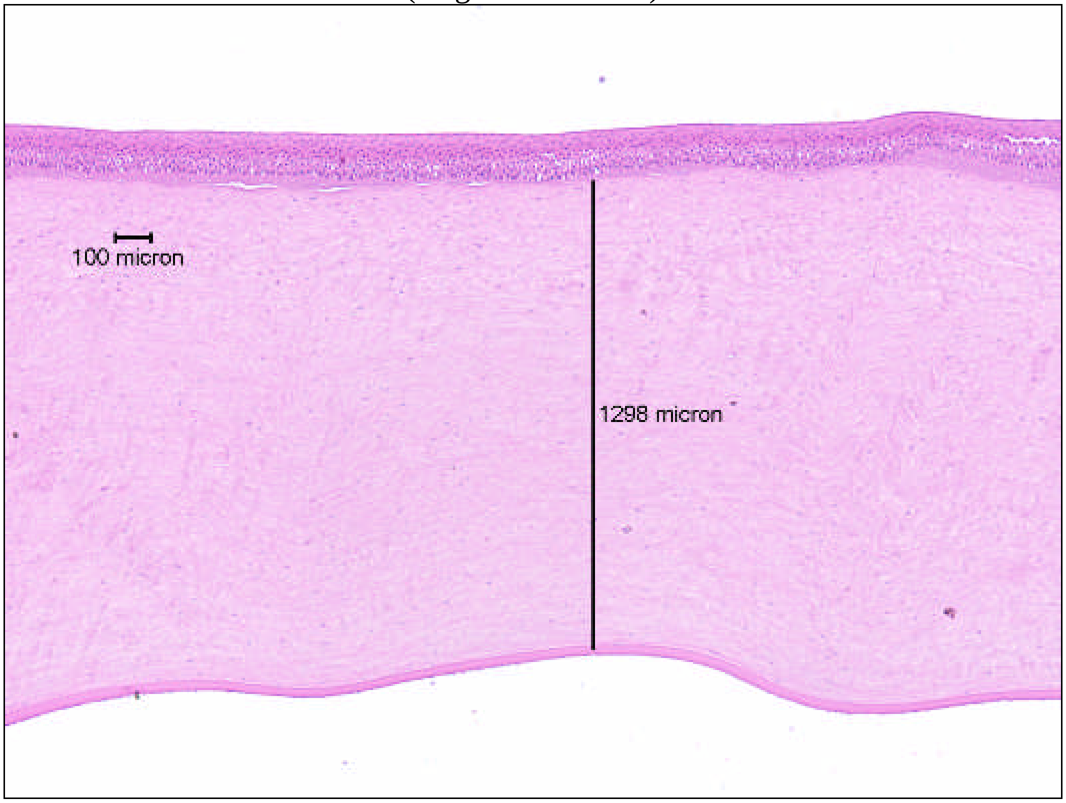


Figure 16. C, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma at 20% depth showing moderate to marked collagen matrix vacuolization and a marked increase in the frequency of keratocytes with hyper-condensed nuclei (magnification 475x)

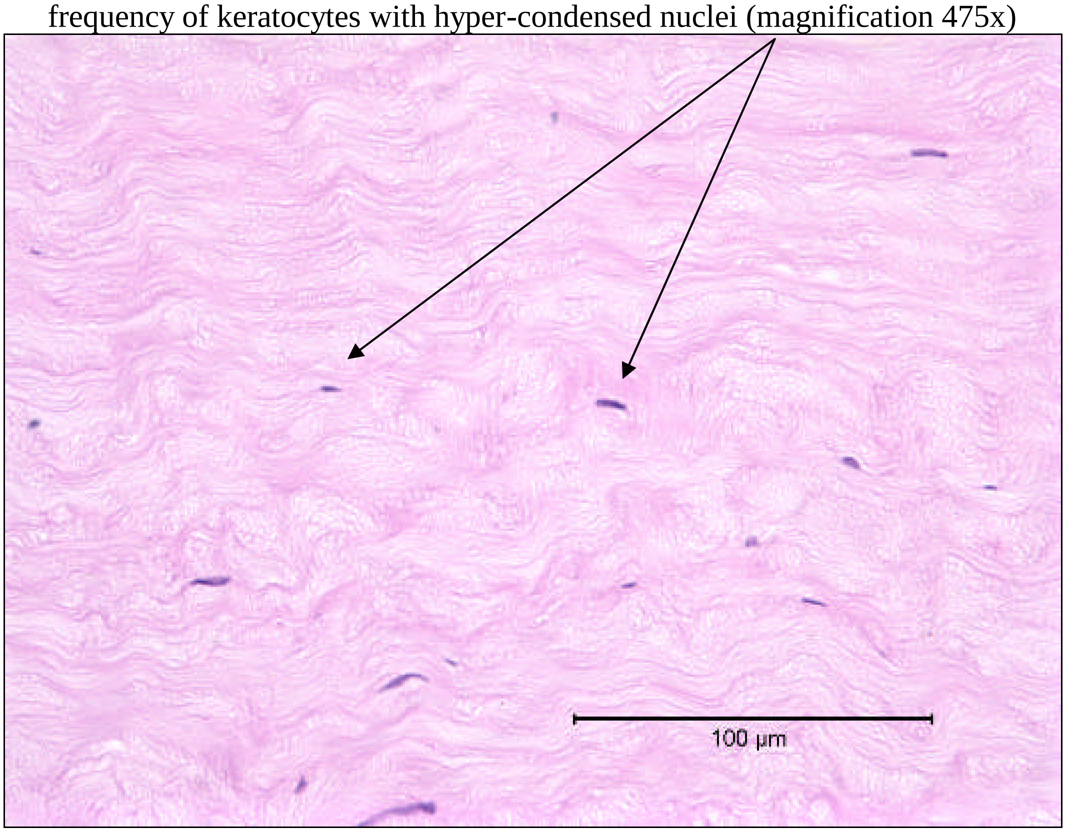


Figure 17. C, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma below mid depth showing moderate to marked collagen matrix vacuolization and a marked increase in the frequency of keratocytes with hyper-condensed nuclei (magnification 475x)

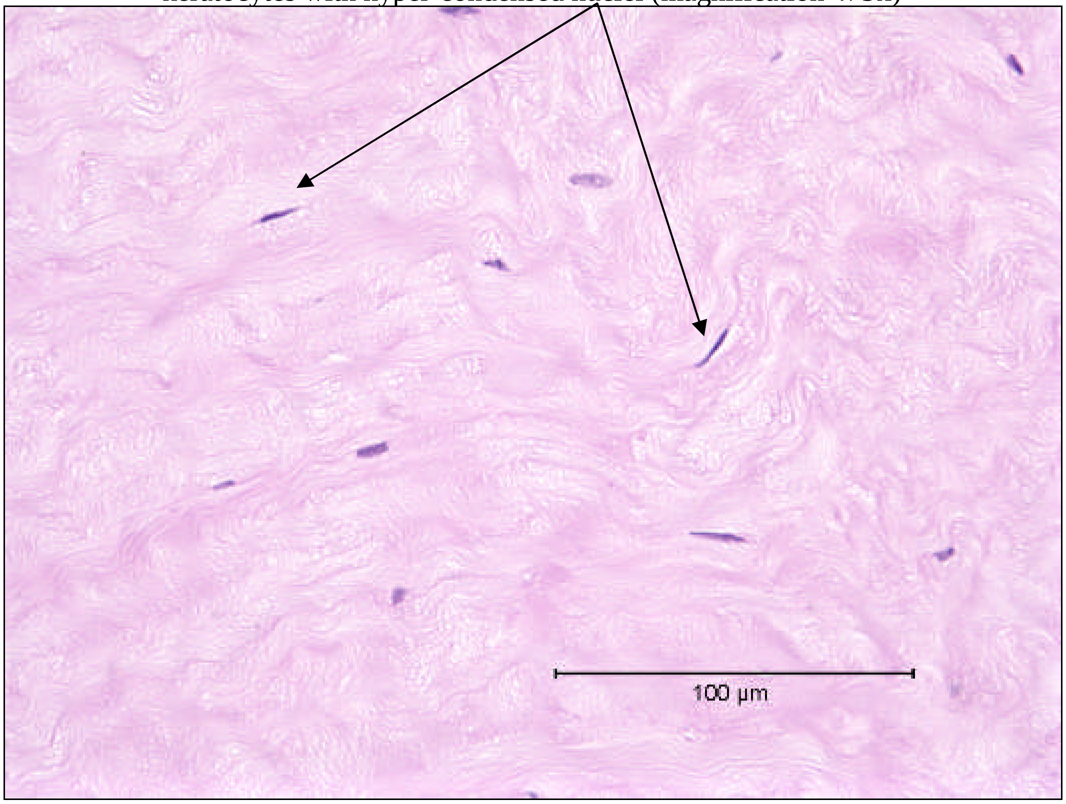


Figure 18. H, neat, 3-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium (magnification 237x)

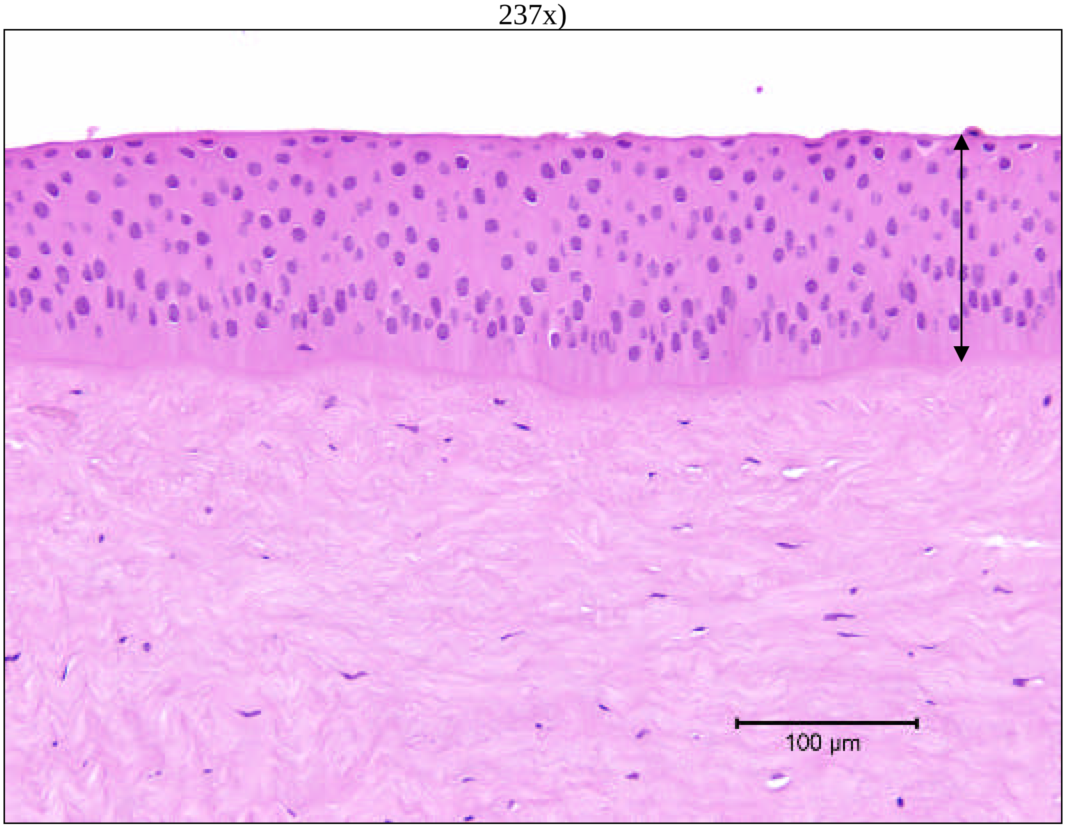


Figure 19. H, neat, 3-minute exposure, 120-minute post-exposure (11/02/05) - Full thickness (magnification 48x)

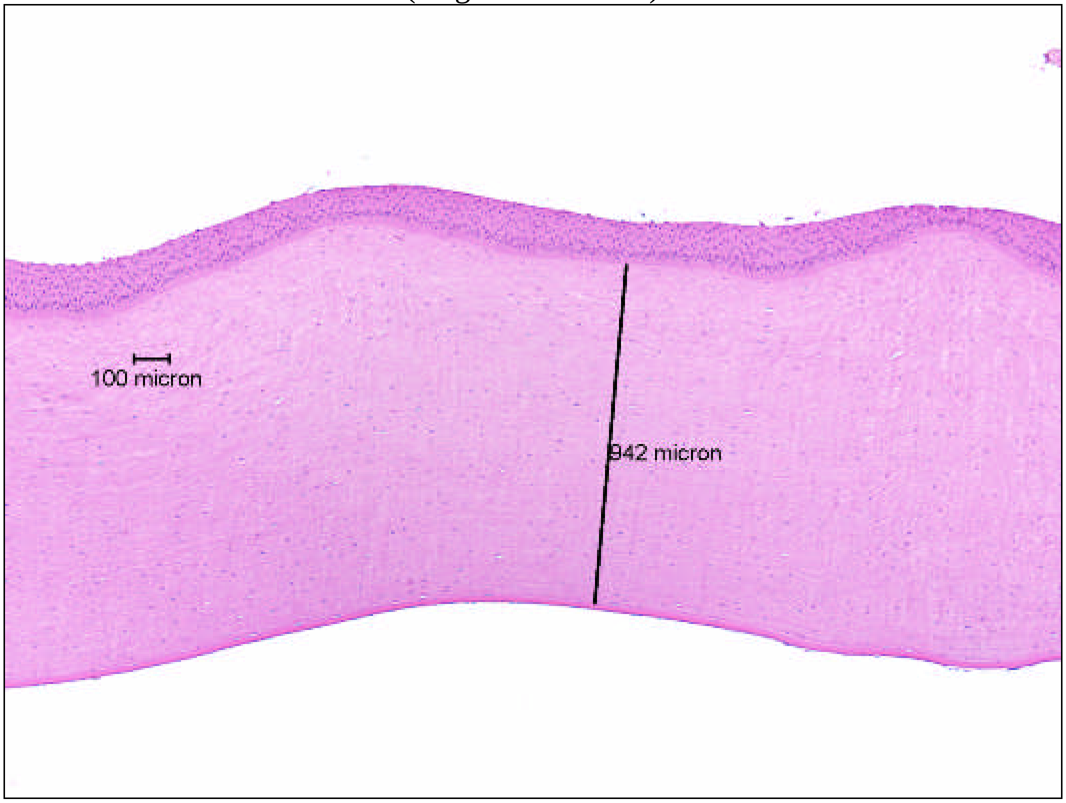


Figure 20. H, neat, 3-minute exposure, 120-minute post-exposure (11/02/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and a moderate increase in keratocytes with abnormal nuclear condensation (magnification 475x)

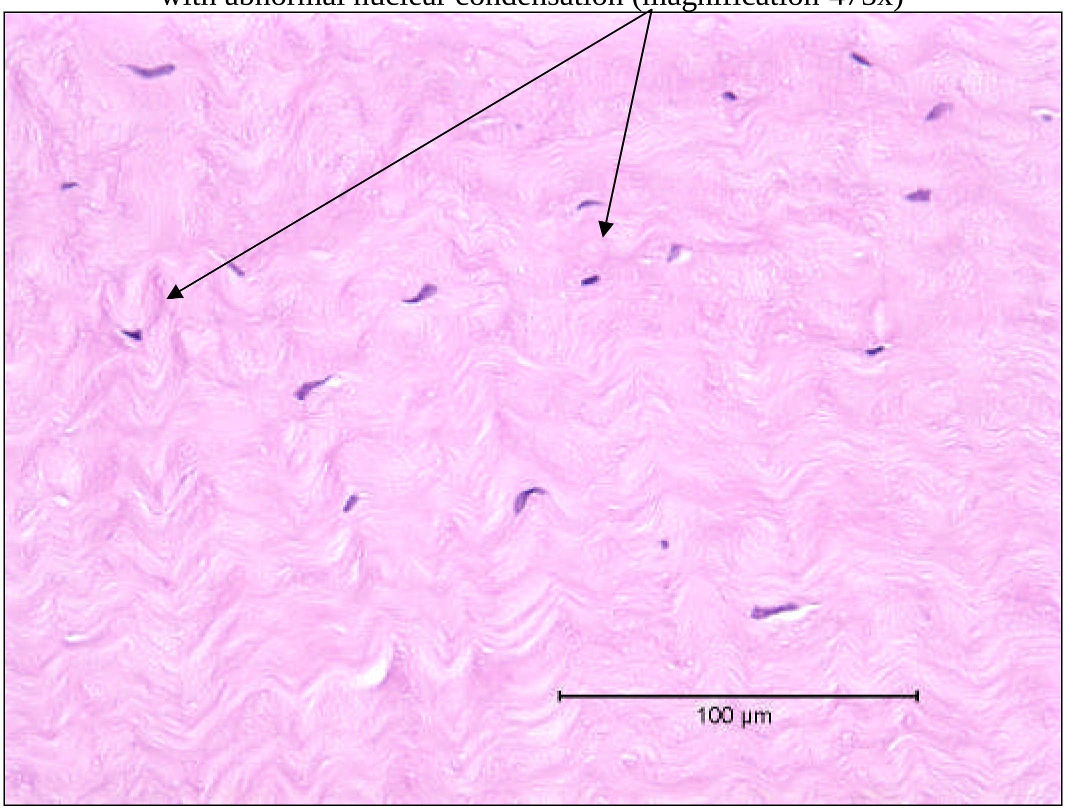


Figure 21. H, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium (overview) (magnification 237x)

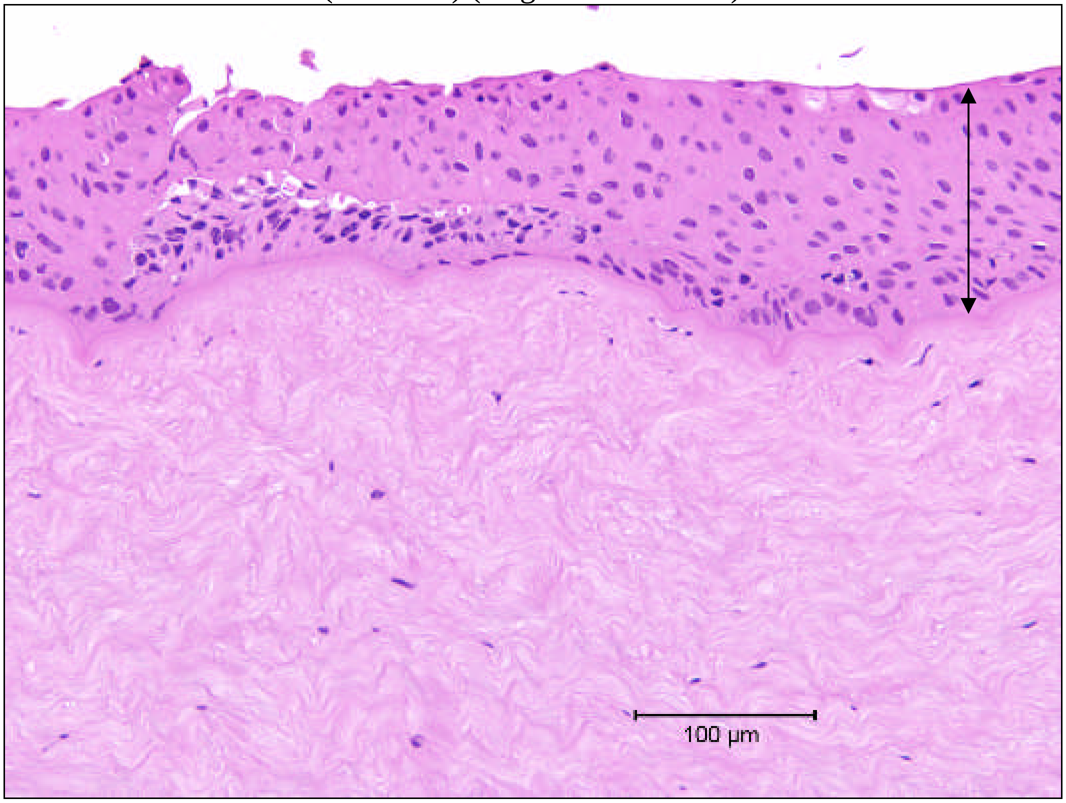


Figure 22. H, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium showing the loss of the squamous cell layer, nuclear and cytoplasmic changes in the wing cells and marked degradation of the basal cells (magnification 475x)

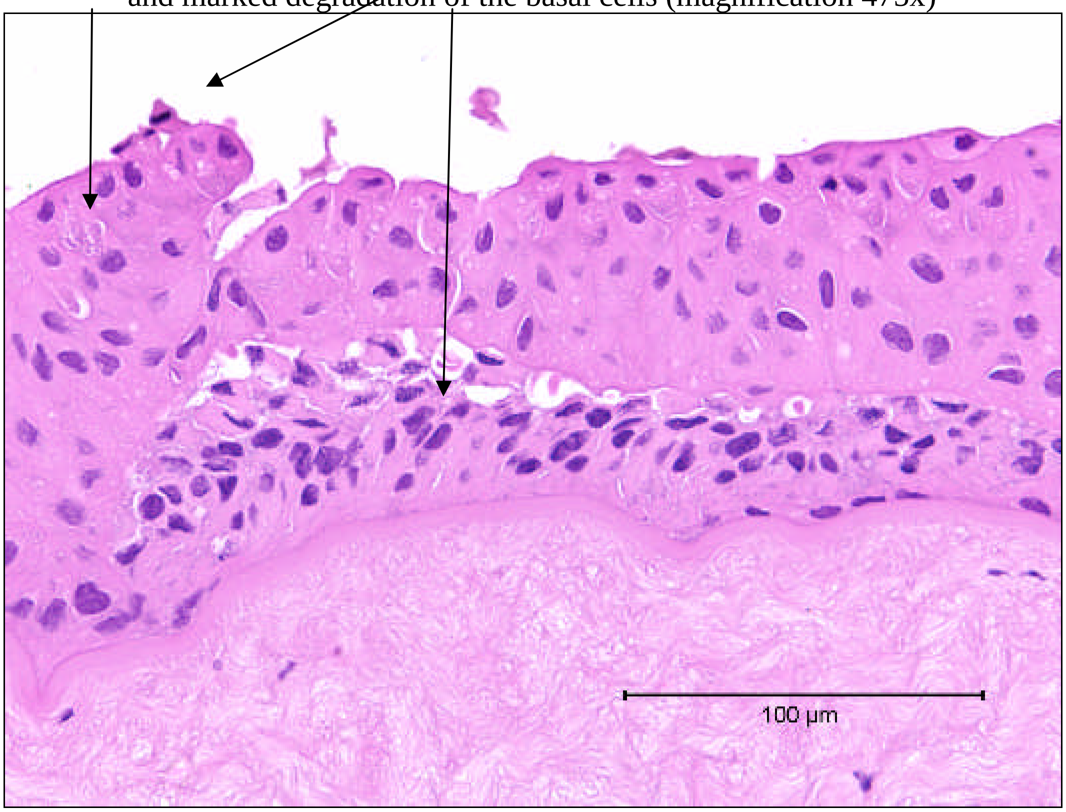


Figure 23. H, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Epithelium showing a full thickness lesion of the epithelium (magnification 237x)

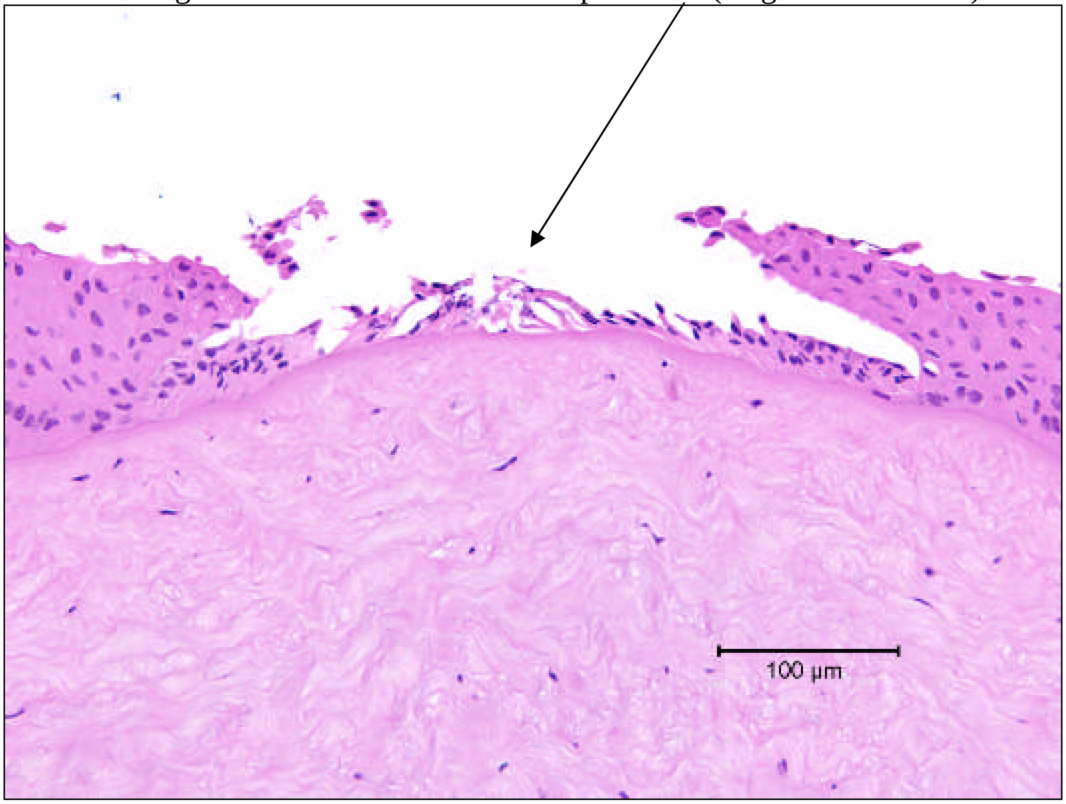


Figure 24. H, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Full thickness (magnification 48x)

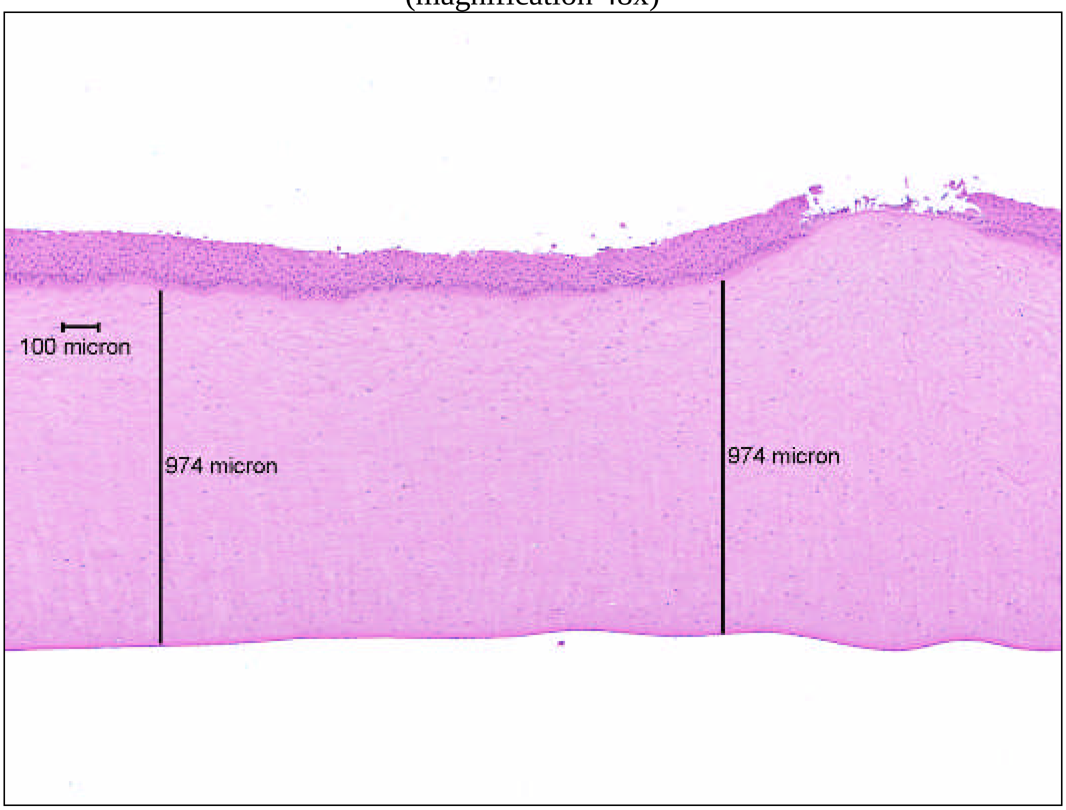


Figure 25. H, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma at 20% depth showing moderate collagen matrix vacuolization and a marked increase in keratocytes with hyper-condensed nuclei (magnification 475x)

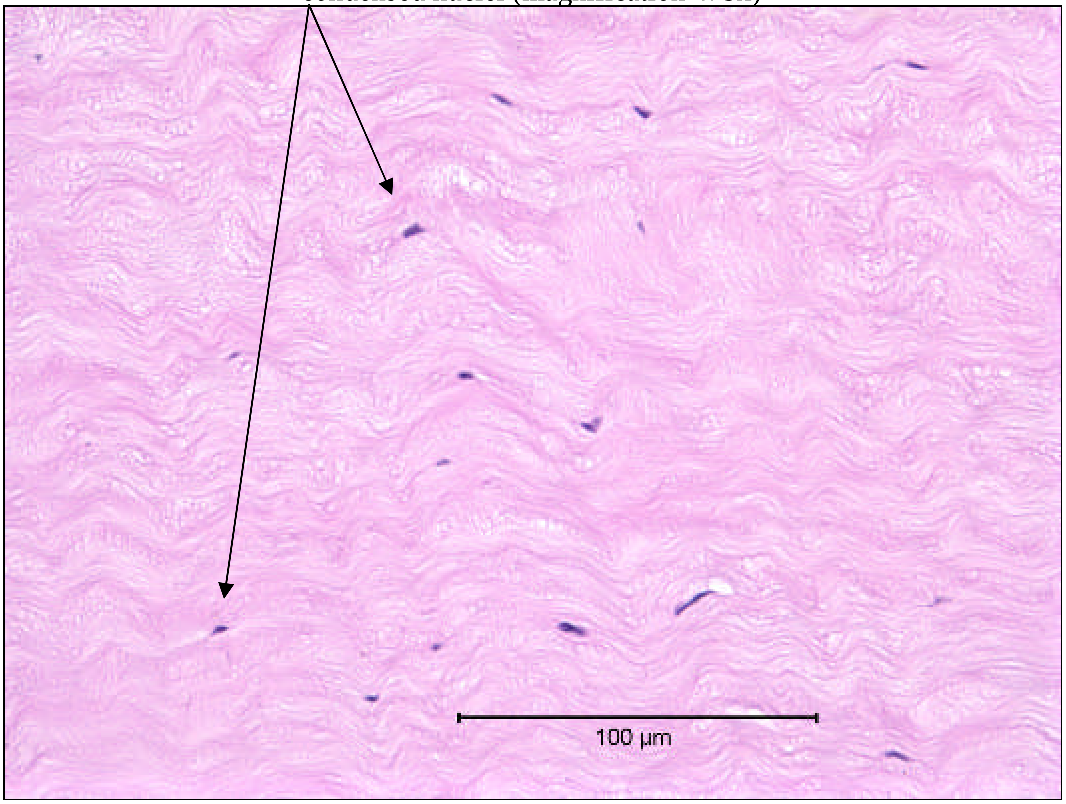
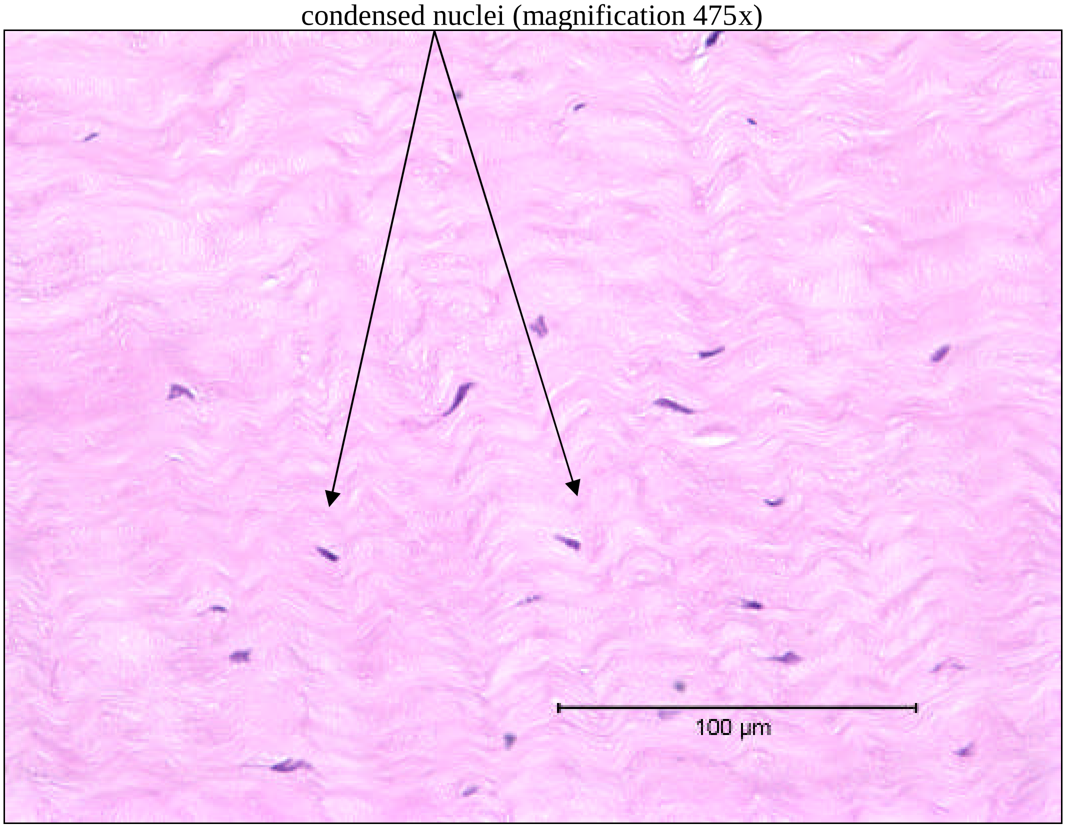


Figure 26. H, neat, 10-minute exposure, 120-minute post-exposure (11/02/05) - Stroma below mid depth showing moderate collagen matrix vacuolization and a marked increase in keratocytes with hyper-condensed nuclei (magnification 475x)



APPENDIX A

APPENDIX B

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

TA #	CORNEA #	INITIAL	FINAL	CHANGE	CORRECTED	AVG	STDEV
05AG40	10	5	7	2	2.3		
Neat	11	3	4	1	1.3		
3 minutes	12	5	8	3	3.3	2.3	1.0
05AG40	14	2	9	7	7.3		
Neat	15	4	11	7	7.3		
10 minutes	16	5	10	5	5.3	6.7	1.2
05AG41	17	3	3	0	0.3		
Neat	18	3	3	0	0.3		
3 minutes	19	4	3	-1	-0.7	0.0	0.6
05AG41	21	4	4	0	0.3		
Neat	22	3	3	0	0.3		
10 minutes	24	3	3	0	0.3	0.3	0.0
05AG42	25	4	9	5	5.3		
Neat	28	4	12	8	8.3		
3 minutes	29	4	13	9	9.3	7.7	2.1
05AG42	32	4	29	25	25.3		
Neat	35	4	42	38	38.3		
10 minutes	37	4	36	32	32.3	32.0	6.5
05AG43	38	3	8	5	5.3		
Neat	44	4	11	7	7.3		
3 minutes	45	3	8	5	5.3	6.0	1.2
05AG43	47	5	21	16	16.3		
Neat	48	4	20	16	16.3		
10 minutes	49	4	27	23	23.3	18.7	4.0
05AE40	40	5	5	0	0.3		
Neat	41	4	5	1	1.3		
3 minutes	43	4	4	0	0.3	0.7	0.6
05AE40	50	5	6	1	1.3		
Neat	51	4	7	3	3.3		
10 minutes	52	4	7	3	3.3	2.7	1.2
Neg. Control	1	3	3	0	NA		
Sterile, DI water	2	3	3	0	NA		
10 minutes	3	4	3	-1	NA	-0.3	
Pos. Control	4	4	29	25	25.3		
Ethanol	6	2	30	28	28.3		
10 minutes	8	3	28	25	25.3	26.3	1.7
	*23	4					
	*42	3					
	*53	4					
	*54	5					

Initial corneal opacity average: 4

* - Corneas not used in this assay, but used to find initial opacity average.
NA - Not Applicable

PERMEABILITY SCORE

Neg. Control
Sterile, DI water
10 minutes

Cornea #	OD490
1	0.005
2	0.003
3	0.004
Avg.	0.004

Pos. Control
Ethanol
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
4	1.407	1	1.403
6	1.208	1	1.204
8	1.405	1	1.401
		Avg. =	1.336
		STDEV=	0.114

05AG40
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
10	0.019	1	0.015
11	0.028	1	0.024
12	0.013	1	0.009
		Avg. =	0.016
		STDEV=	0.008

05AG40
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
14	0.034	1	0.030
15	0.036	1	0.032
16	0.052	1	0.048
		Avg. =	0.037
		STDEV=	0.010

05AG41
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
17	0.002	1	-0.002
18	0.003	1	-0.001
19	0.001	1	-0.003
		Avg. =	-0.002
		STDEV=	0.001

05AG41
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
21	0.003	1	-0.001
22	0.007	1	0.003
24	0.003	1	-0.001
		Avg. =	0.000
		STDEV=	0.002

05AG42
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
25	0.217	1	0.213
28	0.779	1	0.775
29	0.543	1	0.539
		Avg. =	0.509
		STDEV=	0.282

05AG42
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
32	1.338	1	1.334
35	1.285	1	1.281
37	0.427	5	2.131
		Avg. =	1.582
		STDEV=	0.476

05AG43
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
38	0.259	1	0.255
44	0.400	1	0.396
45	0.277	1	0.273
		Avg. =	0.308
		STDEV=	0.077

05AG43
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
47	0.464	1	0.460
48	0.628	1	0.624
49	1.128	1	1.124
		Avg. =	0.736
		STDEV=	0.346

05AE40
Neat
3 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
40	0.133	1	0.129
41	0.134	1	0.130
43	0.166	1	0.162
		Avg. =	0.140
		STDEV=	0.019

05AE40
Neat
10 minutes

Cornea #	OD490	Dilution Factor	Corrected OD490
50	0.915	1	0.911
51	0.429	1	0.425
52	0.903	1	0.899
		Avg. =	0.745
		STDEV=	0.277

IN VITRO SCORE

In Vitro Score = Mean Opacity Value + (15 x Mean OD490)

Test Article	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
05AG40	Neat	3 minutes	2.3	0.016	2.6
05AG40	Neat	10 minutes	6.7	0.037	7.2
05AG41	Neat	3 minutes	0.0	-0.002	0.0
05AG41	Neat	10 minutes	0.3	0.000	0.3
05AG42	Neat	3 minutes	7.7	0.509	15.3
05AG42	Neat	10 minutes	32.0	1.582	55.7
05AG43	Neat	3 minutes	6.0	0.308	10.6
05AG43	Neat	10 minutes	18.7	0.736	29.7
05AE40	Neat	3 minutes	0.7	0.140	2.8
05AE40	Neat	10 minutes	2.7	0.745	13.8
Ethanol	Neat	10 minutes	26.3	1.336	46.4